The influence of temperature on the effects induced by Triclosan and Diclofenac in mussels

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HIGHLIGHTS

- Mussels lowered their metabolism when exposed to TCS or DIC, regardless temperature.
- Mussels increased antioxidant defences when exposed to TCS and DIC, regardless temperature.
- Exposure to DIC at higher temperature enhanced lipid peroxidation.
- Oxidative stress was evidenced in mussels exposed to TCS and DIC.
- Impacts of TCS and DIC were not enhanced under increased temperature.

GRAPHICAL ABSTRACT
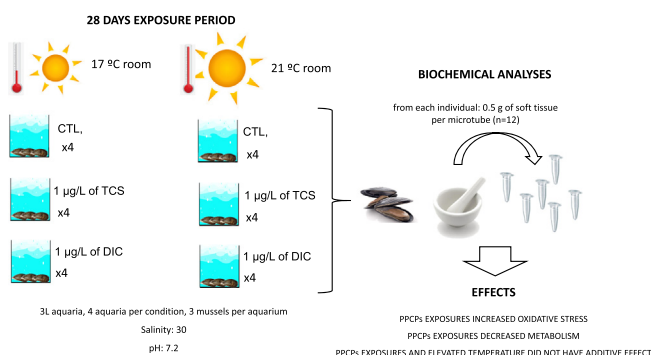
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ABSTRACT

Little is known about the consequences of exposure to pharmaceuticals and personal care products (PPCPs) in elevated temperatures associated with climate change. To increase the knowledge on this topic, *Mytilus galloprovincialis* mussels were exposed to 1.0 μg/L of either the antimicrobial Triclosan (TCS) or the anti-inflammatory drug Diclofenac (DIC), at control (17 °C) and 4 °C raised (21 °C) temperatures for 28 days. Triclosan and DIC concentrations in the water and tissues were subsequently measured and related to biomarker responses including: energy metabolism (electron transport system (ETS) activity, glycogen and protein reserves), oxidative stress markers, glutathione balance between the reduced and the oxidised form (GSH/GSSG), and damage to proteins and lipids. Mussels responded to the increase in temperature and drug exposure by lowering their metabolic rate (decreased ETS), increasing their endogenous reserves and antioxidant defences, thus preventing oxidative stress damage, with the exception of DIC exposure at the higher temperature. In all cases, GSH/GSSG ratio was reduced in detriment of the antioxidant form at both PPCPs exposures and elevated temperature with no additive effect due to combined stressors. Overall, either drug exposure or increased temperature could compromise the ability of mussels to withstand further insults.

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1. Introduction

A wide range of substances considered to be Contaminants of Emerging Concern (CECs) by the US Environmental Protection Agency (EPA) or identified as Emerging Substances by the EU NORMAN network, are not new chemicals and have been present in the environment for a long time. Nonetheless, their presence in water bodies and ecotoxicological impact on aquatic species has only recently been questioned (Fabbri and Franzellitti, 2016). The ubiquity of these substances in the aquatic environment and the limited information regarding their potential toxicity in non-target species, as well as the development of modern analytical methods for trace determinations, explain the new concern over these contaminants (Crane et al., 2006). Among the CECs are pharmaceuticals and personal care products (PPCPs), such as Triclosan (TCS) and Diclofenac (DIC). Triclosan is an antibacterial and antifungal agent found in several products, including toothpaste, soaps, detergents, and surgical cleaning treatments, with concentrations ranging from the scale of ng/L in natural aquatic systems to µg/L in wastewaters (Pintado-Herrera et al., 2014; Dhillon et al., 2015; Montaseri and Forbes, 2016; Olaniyan et al., 2016). Diclofenac is a nonsteroidal anti-inflammatory and analgesic drug used to reduce inflammation and pain, appearing in surface waters and wastewater effluents with concentrations ranging from several ng/L to a few mg/L (Vieno and Sillanpää, 2014; Lonappan et al., 2016; Bonnefille et al., 2017; Cunha et al., 2017). The widespread use of both of these PPCPs has contributed to their presence in a diverse range of aquatic ecosystems which, even at low concentration levels, can lead to adverse effects in wildlife. A moderate body of evidence on the toxic effects induced by TCS and DIC in bivalves has been already published. Among the effects are impairments on biochemical and physiological performance in the clam *Ruditapes philippinarum* (Matozzo et al., 2012; Munari et al., 2018), in the estuarine mussels *Mytilus galloprovincialis* and *Mytilus edulis* (Canesi et al., 2007; Ericson et al., 2010; Gatidou et al., 2010; Gonzalez-Rey and Bebianno, 2014; Cunha et al., 2017; Mezzelani et al., 2018; Munari et al., 2018; Tato et al., 2018), as well as in the freshwater mussels *Perna perna* and *Dreissena polymorpha* (Binelli et al., 2011; Cortez et al., 2012; Pusceddu et al., 2018; Quinn et al., 2011; Riva et al., 2012).

Although significant advances on the impact of PPCPs on aquatic organisms have been made, uncertainties still remain regarding the consequences of these exposures in combination with sustained modifications caused by Climate Change (CC) related factors. In particular, CC-induced environmental alterations, such as temperature increases due to extreme weather events (Gazeau et al., 2013; Intergovernmental Panel on Climate Change - IPCC, 2014), are likely to significantly affect organisms (Freitas et al., 2017a; Freitas et al., 2016), but may also affect the pollutants behaviour and bioavailability and, hence, their bioaccumulation potential and impact. In this regard, scarce information is available considering the combined effects of CC and PPCPs in bivalves. A recent study by Serra-Compte et al. (2018) demonstrated that the expected warming and acidification of seawater modified the bioconcentration of different pharmaceuticals and endocrine disrupting compounds (EDCs) in the mussel *M. galloprovincialis*. Another recent study from Munari et al. (2018) evaluated the combined impact of pH and DIC in *M. galloprovincialis* and *R. philippinarum* oxidative stress status, revealing that the biochemical parameters measured in both species were more influenced by reduced pH than by the contaminant itself or the pH*contaminant interaction, although the biomarker variation patterns differed depending on the species and tissues that were analysed. Gonzalez-Ortegon et al. (2013) studied the effects of three common pharmaceuticals (DIC, Clofibric acid and Clotrimazole) on growth, development and body mass during the larval stages of the marine shrimp *Palaemon serratus* at different temperatures and salinities and demonstrated that the effects of these pollutants were stronger when organisms are under additional environmental stress.

The use of biomarkers indicative of sublethal responses that are induced by PPCPs and temperature in terms of metabolic and oxidative stress in bivalves has already been proven to be adequate. Metabolic capacity was evaluated by measuring the electron transport system activity, as it had previously been shown that this parameter responded to warming conditions (Le Moullac et al., 2007; Freitas et al., 2017a; Coppola et al., 2018) and the presence of pollutants (Andrade et al., 2018; Freitas et al., 2018; Monteiro et al., 2019). The oxidative stress response of mussels was evaluated by assessing different biomarkers related to antioxidant defence and cellular damage. Different xenobiotics, including PPCPs, are capable of inducing oxidative stress in aquatic organisms, through the over-production of reactive oxygen species (ROS), and associated stress damage. In order to avoid an imbalance in their oxidative status, aquatic organisms have the capacity to trigger antioxidant defences, that involve the activation of specific antioxidant enzymes, such as superoxide dismutase and catalase. When the activity of antioxidant and biotransformation enzymes are not effective in preventing the establishment of an oxidative stress condition, the excess of ROS may induce oxidative damage, including lipid peroxidation of biological membranes and oxidised protein formation (Regoli and Giuliani, 2014).

The aim of the present study was to assess the influence of temperature on the toxicity induced by PPCPs in mussels, testing the hypotheses that the biochemical performance of mussels is not affected by exposure to TCS or DIC; and that the impacts of PPCPs in mussels were not influenced by temperature. Adult specimens of *M. galloprovincialis* were independently exposed for a period of 28 days to either TCS or DIC (1 µg/L) in two water temperature conditions (17 and 21 °C). Chemical analysis of water and tissue levels of the PPCPs was measured and the mussel's biochemical performance was evaluated using energy metabolism and oxidative stress parameters.

2. Materials and methods

2.1. Experimental conditions

Mytilus galloprovincialis were collected during low tide in a subtidal area located at the Mira Channel (Ria de Aveiro, a coastal lagoon, northwest of Portugal), in September 2017. After sampling, the specimens were placed in aquaria for depuration and acclimation to laboratory conditions for 7 days. Artificial seawater (salinity 30 ± 1), made with artificial salt (Tropic Marin®SEA SALT from Tropic Marine Center) and deionized water, was used. During this period the organisms were maintained at 17 ± 1.0 °C, salinity 30 ± 1.0 and pH 8.0 ± 0.1, resembling conditions at the sampling area during their collection, and kept under continuous aeration during a 12 h light: 12 h dark photoperiod. During this period seawater was renewed 2–3 times per week and organisms were fed with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc. (150,000 cells/animal).

After acclimation, organisms were distributed in different aquaria (3 L), to test the impacts induced by the exposure to Triclosan (TCS) and Diclofenac (DIC) under different temperature values, testing the following 6 conditions: CTL, 0 µg/L PPCPs at temperatures 17 or 21 °C; TCS, 1 µg/L of TCS and 0 µg/L of DIC at temperatures 17 or 21 °C; DIC, 1 µg/L DIC of and 0 µg/L of TCS at temperatures 17 or 21 °C. Triclosan (CAS: 3380-34-5. REF: PHR1338) and DIC (CAS: 15307-86-5. REF: D6899) were purchase from Sigma-Aldrich and used to prepare the stock solutions.

Per condition 4 aquaria were used, with 3 organisms in each. During the experiment water samples from the control and contaminated tanks were collected immediately after spiking to ensure chemical concentration. At the end of the exposure period (28 days) TCS and DIC concentrations were also determined in mussels whole soft tissue.

During the experimental period (28 days) mussels were maintained at constant aeration. Temperature (17 or 21 °C) and salinity (30) were daily checked and readjusted if necessary. Along the exposure period, mussels were fed with AlgaMac Protein Plus twice a week, as described

above, and seawater was renewed weekly, after which the experimental conditions were re-established, including seawater parameters and PPCPs concentrations to ensure the same exposure concentrations during the experiment. Along the experiment no mortality was observed.

After exposure, with the objective to integrate the general health status of each individual, the whole soft tissues of each organism were individually frozen and manually homogenized with a mortar and a pestle under liquid nitrogen. Each homogenized organism was divided into aliquots of 0.5 g fresh weight (FW), which were used for biomarkers analyses and PPCPs quantification.

2.2. Triclosan and Diclofenac determinations

The TCS concentrations in water samples were obtained following the procedure reported by Cheng et al. (2011). The extraction was performed with C-18 SPE cartridges (HYPERSEP, 6 mL, 1 g, Thermo Scientific). Each cartridge was pre-conditioned with 6 mL of dichloromethane, and 6 mL of methanol and then rinsed with 6 mL of ultrapure water by the use a SPE manifold (Thermo Scientific). The samples were acidified to pH 3 (Cheng et al., 2011), and passed through the cartridges with the aid of the vacuum. Then, the cartridges were washed with 4 mL 20% methanolic solution and air-dried under vacuum for 20 min. TCS was eluted from the cartridges with 8 mL of dichloromethane and the extracts were completed dried with rotavapor. The residue was redissolved in 0.2 mL of toluene and analysed by GC–MS. Triclosan was extracted from soft tissues as suggested by Schmidt and Snow (2016) by using a QuEChERS method. Ultrapure water was added to an aliquot of sample tissue into a 50 mL polypropylene tube and the mixture was manually shaken. Acetonitrile was successively poured and the tube was still shaken by hand. Then, QuEChERS extraction salts kit was added and the extraction mixture was vigorously shaken again. The centrifuged extract was finally treated with QuEChERS purification salts kit, filtered and concentrated in a 2 mL vial for GLC analysis. The extracts were then processed according to the GC–MS method reported by Tohidi and Cai (2015). Triclosan solid standard was purchased from Sigma-Aldrich. Standard stock solutions were prepared in dichloromethane (concentration range: 0.1–1.2 mg/L). All the stock solutions were stored at <6 °C. The QuEChERs extraction kit tubes were purchased from Agilent Technologies. Triclosan analyses were performed with a GC Trace 1300 (Thermo Scientific) coupled to a TriPlus RSH autosample and a triple quadrupole mass spectrometer TSQ Duo with an electron impact ionization source (EI) (Thermo Scientific). The GC was equipped with an Agilent DB-5MS column. The analysis was performed in SIM technique. Highly pure helium was used as carrier gas for the GC analyses. The limit of detection (LOD), calculated as a signal-to-noise ratio of 3:1, was 0.008 μg/L for water samples and 0.13 ng/g dry weight (d.w) for soft tissues. The recovery was >91% for water samples and >89% for soft tissues.

Concentrations of DIC were measured in water and soft tissues by using a high performance liquid chromatography-ultraviolet (HPLC-UV) detection method. Water samples were analysed by using the method of Madikizela and Chimuka (2017) with slightly modifications. Water samples (100 mL) were extracted by solid phase extraction (Oasis MAX 6cc 150 mg solid-phase extraction cartridges, Waters). The pH of water samples was adjusted to 2.5, then 100 mL of each sample was loaded onto a pre-conditioned cartridge. The SPE cartridge was rinsed with methanol: water (10:90%, v:v) prior to sequential elution of retained DCF with 2 mL methanol, followed by 2 mL methanol and acetic acid (90:10, v:v) and 2 mL of 2% (v:v) formic acid diluted using a mixture of methanol and acetic acid (40:60, v:v). The eluted analytes from the SPE cartridge were quantified using HPLC. Diclofenac in soft tissues samples were analysed by using the method of Gatidou et al. (2007) with slightly modifications. Whole tissue samples of 3 individuals (1.5 g) were dehydrated and sonicated at 50 °C for 30 min using 16 mL of mixture of methanol (10 mL) and Milli-Q water (6 mL) as the extraction solvent. The supernatant was collected after

centrifugation and diluted to a final volume of 100 mL using Milli-Q grade water and then purified with solid phase extraction as reported for water samples. The chromatographic system consisted of a Series 200 PerkinElmer gradient pump coupled to a Series 200 PerkinElmer variable UV detector, which was set at 280 nm. The mobile phase consisted of acetonitrile and 0.2% formic acid in water, at a ratio of 60:40 (v:v). A 100 μL injection was used each time. The reversed-phase column was a Haisil, LC column (5 μm, 150 × 4.60 mm, Higgins). The column was kept at room temperature. Turbochrome software was used for data processing. The recovery was >80% for water samples and >77% for soft tissues. The LOD, calculated as a signal-to-noise ratio of 3:1, was 0.10 μg/L for water samples and 5 ng/g d.w for soft tissues.

2.3. Biochemical parameters

After 28 days exposure, mussels used for biomarker analysis (2 per replicate, 8 per condition) were extracted with specific buffers for each biomarker in the proportion 1:2 w/v (Almeida et al., 2014; Andrade et al., 2018, 2019; De Marchi et al., 2018). For each biochemical determination, 0.5 g FW of soft tissue per organism was used. For each condition indicators of metabolic capacity (electron transport system activity, ETS), energy reserves (total protein content, PROT; glycogen content, GLY), oxidative stress (including, lipid peroxidation levels, LPO; Protein carbonylation levels, PC; ratio reduced (GSH)/oxidised (GSSG) glutathione, superoxide dismutase activity, SOD; catalase activity, CAT; glutathione S-transferases activity, GSTs) were determined. The samples were homogenized for 15 s at 4 °C and centrifuged for 20 min at 10,000g (or 3,000 g for ETS) at 4 °C. Supernatants were stored at −80 °C or immediately used. All biochemical parameters were performed in duplicate. All measurements were done using a microplate reader (Biotek).

2.4. Data analysis

All the biochemical results (ETS, GLY, PROT, SOD, CAT, GSTs, LPO, PC, GSH/GSSG) were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7). A one-way hierarchical design was followed in this analysis. The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. Pairwise comparisons were performed when significant differences were observed in the main test. Values lower than 0.05 were considered as significantly different. The null hypothesis tested was: for each biomarker, no significant differences existed among exposure conditions (CTL 17 °C, TCS 17 °C, DIC 17 °C, CTL 21 °C, TCS 21 °C, DIC 21 °C). Significant differences among exposure conditions were represented with different letters.

3. Results and discussion

3.1. Triclosan and Diclofenac concentrations

Uncontaminated seawater (control conditions) contained PPCPs concentrations that were lower than the limit of detection (LOD) for Diclofenac (DIC, 0.10 μg/L) and Triclosan (TCS, 0.008 μg/L), ensuring the quality of the water that was used in the experiment. The TCS water concentrations were similar for both temperatures soon after spiking (0.74 ± 0.10 μg/L at 17 °C and 0.84 ± 0.19 μg/L at 21 °C). The DIC seawater concentrations ranged between 0.96 ± 0.05 μg/L (17 °C) and 0.91 ± 0.03 μg/L (21 °C) immediately after spiking. These results indicate that after spiking with TCS or DIC (1 μg/L), the former is less stable in the medium while DIC keeps concentrations that are closer to the initial value (Table 1). We are not aware of any previous studies that report TCS water concentrations. In regard to DIC natural water concentrations, these have been previously reported at similar levels to ours in studies that used concentrations similar to the ones used in the present experiment (Fontes et al., 2018; Schmidt et al., 2014). However, in

Table 1
Laboratory studies in adult bivalves after water exposures to Triclosan (TCS) and Diclofenac (DIC). Limit of detection (LOD) in water in µg/L is 0.008 (TCS) and 0.10 (DIC) and in tissue in ng/g d.w is 0.13 (TCS) and 5.0 (DIC). Results from the present study are in bold.

Chemical	Nominal water conc (µg/L)	Measured water conc (µg/L)	Bivalve species	Length exposure days	Tissue concentr ng/g d.w	Reference
Triclosan (TCS)	**1.0**	**0.64–1.03**	**_M. galloprovincialis_**	**28**	**<LOD-0.66**	**This study**
	0.3	n.a	_M. galloprovincialis_	28	881	Gatidou et al., 2010
	0.1, 1	n.a	_M. galloprovincialis_	14	≈100	Kookana et al., 2013
	0.1	n.a		30	40	
	0.3, 0.6, 0.9	n.a	_R. philippinarum_	7	n.a	Matozzo et al., 2012
	0.3, 0.6, 0.9		_D. polymorpha_	3	n.a	Binelli et al., 2011
	0.58	n.a	_D. polymorpha_	7	248 ± 45	Riva et al., 2012
Diclofenac (DIC)	**1.0**	**0.88–1.01**	**_M. galloprovincialis_**	**28**	**29–84**	**This study**
	0.5	n.a	_M. galloprovincialis_	14	4.75 ± 5.3	Mezzelani et al., 2016
	2.5	n.a	_M. galloprovincialis_	14, 30, 60	1.63–3.63	Mezzelani et al., 2018
	0.05, 0.5	n.a	_M. galloprovincialis_ & _R. philippinarum_	14, 21	n.a	Munari et al., 2018
	0.02, 0.2, 2.0	0.02, 0.21, 2.2	_Perna Perna_	2	n.a	Fontes et al., 2018
	1, 1000	0.60–0.87, 80–410	_Mytilus_ spp.	7, 10, 14	n.a	Schmidt et al., 2014
	1		_M. edulis trossulus_	8	36 ± 4[a]	Ericson et al., 2010
	0.25		_M. galloprovincialis_	15	n.a	Gonzalez-Rey and Bebianno, 2014

[a] Wet weight/dry weight conversion factor of 5 (Alvarez-Muñoz et al., 2018).

the Fontes et al. (2018) study, an abrupt 96% decrease in water concentration of DIC was observed after 24 h. Despite this, very limited laboratory studies provide confirmation of the selected nominal concentrations in water after exposure to TCS and DIC (Table 1).

Bioaccumulation of TCS and DIC was assessed at the end of the exposure period (28 days). Our results revealed that temperature had no clear influence on PPCPs accumulation, as similar concentrations were found in mussels for both temperatures. Mussels that were exposed to TCS at 17 °C contained concentrations of 0.55 ± 0.11 ng/g d.w, and those exposed to TCS at 21 °C had concentrations close to the LOD. Mussels exposed to DIC showed no significant differences between temperatures, with values ranging between 39 ± 10 ng/g (17 °C) and 44 ± 40 ng/g (21 °C). Comparing both PPCP exposures, lower accumulation values were observed in mussels that were exposed to TCS (<1 ng/g) in comparison to mussels exposed to DIC (10–100 ng/g), regardless of the acclimation temperature. Our results on the bioaccumulation of PPCPs partly differ from other studies that have reported water exposures to the same drugs (Table 1). Triclosan water exposure of 0.3 µg/L in the same bivalve species showed much higher concentrations in tissue (881 ng/g d.w) after a similar length of exposure (Gatidou et al., 2010). Shorter 7-day TCS water exposure of 0.58 µg/L in the freshwater mussel _Dreissena polymorpha_ resulted in 248 ng/g d.w TCS in tissue (Riva et al., 2012). The reason for this discrepancy could be due to the fact that TCS is quickly metabolised to methyl TCS when mussels are present in the medium (Kookana et al., 2013) and in the present study only the parental form was measured. Nonetheless, an equilibrium seems to be reached after 14-days of exposure to a water concentration of 1 µg/L (Kookana et al., 2013). In contrast, in the present study, DIC concentrations measured in the whole tissue of _Mytilus galloprovincialis_ are higher than those reported in the same species using comparable concentrations and length of exposure times (Mezzelani et al., 2016; Mezzelani et al., 2018). Although the present temperature and pH values were similar to those in previous studies, salinity differed between both exposures: 30 (our study) versus 37 (previous studies). Water salinity in the chemical's bioavailability could be partly responsible for these differences. Similar tissue concentrations to those observed in the present study were reported in _Mytilus edulis trossulus_ (≈36 ng/g d.w) after the same water exposure of 1 µg/L (Ericson et al., 2010), shown in Table 1.

The bioccumulated concentrations revealed in the present study are not too distant from tissue concentration attained under natural systems. Triclosan in marine bivalves can also largely fluctuate from a minimum of 1.7 ng/g d.w in clams, _Chamelea gallina_, from the Ebro Delta (NW Mediterranean) (Alvarez-Munoz et al., 2015) to up to 2578 ng/g d.w in mussels _M. galloprovincialis_ in Greece (NE Mediterranean) (Gatidou et al., 2010). An intermediate TCS concentration of 9.87 ± 1.34 ng/g d.w was reported in mussels, _M. galloprovincialis_, caged for up to 70 days at the south coast of Australia where water concentrations fluctuated between 50 and 120 ng/L for TCS at the outlet of two sewage treatment plants (Kookana et al., 2013). With regard to bioccumulated DIC, up to 4.5 ng/g d.w was reached in bivalves at the Portuguese coast (Cunha et al., 2017) while water concentrations ranged from 0.41 to 241 ng/L along the same Atlantic coast (Lolic et al., 2015). On the Atlantic coast of Spain a maximum of 31.9 ng/L DIC was measured (Biel-Maeso et al., 2018). In the Seine estuary surface water presented up to 172.5 ± 32.0 ng/L on the Atlantic coast of France (Togola and Budzinski, 2007). In Mediterranean waters, up to 1.5 µg/L was reached close to urban discharges in the Cortiou inlet (Togola and Budzinski, 2008).

Overall, the present findings demonstrate that water exposure at the high range of environmental concentrations (1 µg/L) resulted in lower TCS and higher DIC being bioccumulated in respect to comparable laboratory exposures and bivalve tissue concentrations in natural systems. However, due to the large variation in water and tissue concentrations reported in bivalves under laboratory and field conditions, it is necessary to include biochemical tools that enable to assess the consequences of the exposures within bivalves' performance.

3.2. Biochemical parameters

The presence of PPCPs in aquatic ecosystems worldwide and their toxic effects on inhabiting organisms has been previously demonstrated. Recently, special attention has been given to the impact that environmental parameters, such as those related to climate change, have on PPCPs toxicity in aquatic invertebrates (Gonzalez-Ortegon et al., 2013; Munari et al., 2018; Serra-Compte et al., 2018). Despite these advances, current understanding of the effect of temperature on PPCPs toxicity is still limited.

3.2.1. Metabolic capacity and energy reserves

The electron transport system activity (ETS) (Fig. 1A) was significantly higher in non-contaminated mussels under 17 °C (control) than those maintained at 21 °C or those exposed to the drugs at either temperature. The glycogen content (GLY) (Fig. 1B) followed the opposite trend to ETS, with significantly lower content in non-contaminated mussels at 17 °C (control) compared to non-contaminated mussels kept at 21 °C or those exposed to drugs at both temperatures. Similarly, the protein content (PROT) (Fig. 1C) was significantly lower in non-contaminated mussels at 17 °C (control) compared to all groups at 21 °C and those exposed to DIC at 17 °C.

The present study clearly demonstrates that the presence of PPCPs or warmer conditions (+4 °C) lead to a significant decrease in the
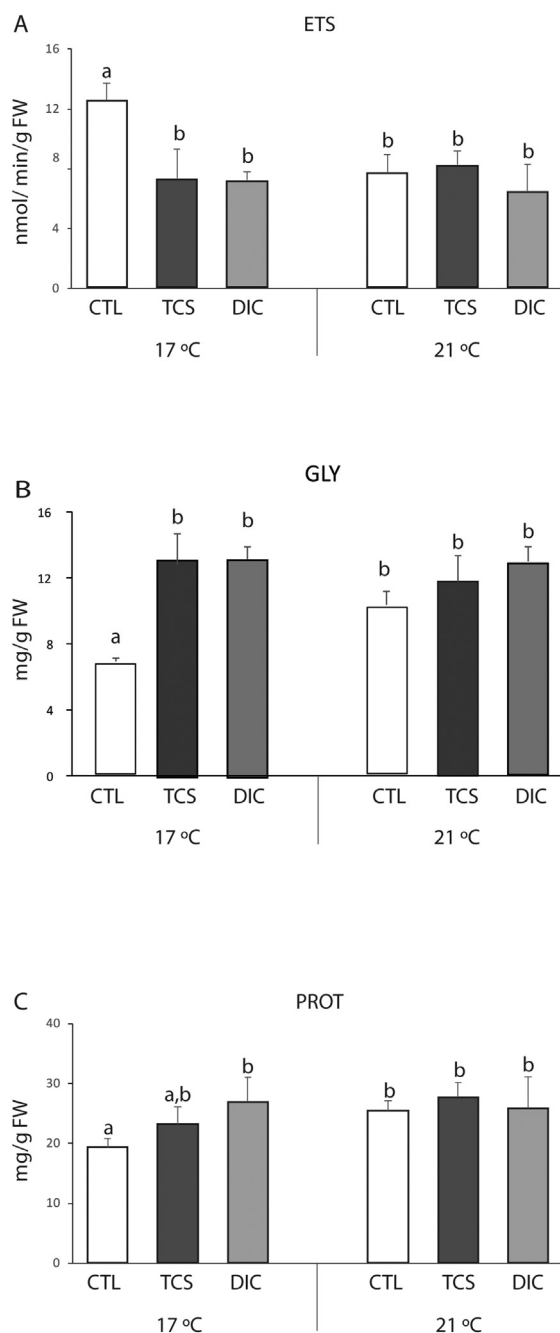
Fig. 1. A: Electron transport system activity (ETS); B: glycogen content (GLY) and C: protein content (PROT), in *Mytilus galloprovincialis* under two temperatures 17 °C (natural) and 21 °C (predicted CC scenario) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 μg/L each. Values are presented as mean + standard deviation. Significant differences (p ≤ 0.05) among treatments are represented with different letters.

metabolic capacity of mussels. This was not enhanced when both stressful conditions were acting in combination (i.e., presence of PPCPs and increased temperature). These findings indicate that by reducing mussel metabolism and energy reserve expenditure, PPCPs can compromise organisms' general performance, by negatively affecting their reproductive capacity, growth rate and abundance. It is well known that physiological processes, such as reproduction, require high energy costs that under such stressful conditions can be greatly affected because organisms must develop mechanisms to protect themselves against the pollutants and increased temperature effects instead of activating strategies for successful reproduction. Nevertheless, effects on bivalve metabolism due to TCS and DIC appear not to be aggravated in a future

global warming scenario. The decreased metabolism observed in *M. galloprovincialis* exposed to PPCPs at both temperatures (17 and 21 °C) was accompanied by an increase in GLY and PROT reserve concentrations in mussels exposed to both stressful conditions. A similar trend, showing ETS activity decrease and energy reserve content increase, was observed in *M. galloprovincialis* at comparable warming conditions (Coppola et al., 2018). Furthermore, in line with our findings, Ericson et al. (2010) also demonstrated that high concentrations of DIC (100 and 1000 μg/L) decreased the respiration rate in blue mussels, which may be a result of metabolism reduction. However, an opposite response was observed in *M. galloprovincialis* which displayed an increase in ETS activity associated with increased energy reserve expenditure upon exposure to the drugs Carbamazepine (Oliveira et al., 2017) and Cetirizine (Teixeira et al., 2017). Together these findings indicate that the response of *M. galloprovincialis* to drugs is highly dependent on the mode of action of each compound. Our results also revealed that an elevated temperature did not have an additive effect on contaminated mussel's metabolism and energy reserve content, indicating that above a certain stress-limit mussels are not able to further reduce their metabolism. The metabolism decrease may result from the ability of bivalves to close their valves and reduce their filtration rate for long periods when they are exposed to stressful conditions, to avoid the accumulation of pollutants. This could explain why similar effects were found at both temperatures in the groups exposed to TCS or DIC. These results of comparable metabolic activity at different temperatures under chemical exposures complement the similar concentrations of TCS and DIC bioaccumulated in mussels kept at either 17 or 21 °C. To our knowledge, this is the first evidence exploring mussel metabolism upon exposure to DIC and TCS under warming conditions.

3.2.2. Antioxidant and biotransformation defences

The activity of superoxide dismutase (SOD) and catalase (CAT) (Fig. 2A and B) displayed no significant differences between non-contaminated mussels at 17 °C and 21 °C, while contaminated mussels showed significantly higher SOD and CAT activities than those considered controls (17 °C non-contaminated group) and those exposed to TCS and higher temperature. These results reveal that oxidative stress occurs in *M. galloprovincialis* mussels exposed to TCS and DIC regardless of the temperature of their environment, demonstrated by the activation of antioxidant enzymes in contaminated organisms independently of the temperature of exposure. In fact, although mussels exposed to TCS and DIC presented a decreased metabolic capacity, they were able to enhance the activity of SOD and CAT antioxidant enzymes in order to eliminate the overproduction of ROS caused by the presence of either PPCP. The present study also demonstrates that increased temperature has no additive effects on a mussel's antioxidant response to PPCPs, as similar activity values were recorded in contaminated mussels at both temperatures. Increased ROS production due to warmer conditions that lead to the activation of antioxidant enzymes, has already been reported in aquatic species (Alves de Almeida and Di Mascio, 2011). Furthermore, in line with the present findings, previous studies have also shown that TCS and DIC induce the activation of antioxidant enzymes in bivalves. That is, Binelli et al. (2011) demonstrated that in the freshwater mussel *D. polymorpha* TCS caused a significant increase in SOD and CAT activity when measured in the whole organism. Whereas, Matozzo et al. (2012) reported that TCS significantly increased SOD activity in the gills but decreased activity in the digestive gland of *Ruditapes philippinarum*. In a study on *M. galloprovincialis* exposed to DIC, SOD activity increased significantly (Gonzalez-Rey and Bebianno, 2014). However, Canesi et al. (2007) showed that injected TCS decreased the activity of CAT in *M. galloprovincialis*, and Munari et al. (2018) reported inconclusive results regarding SOD and CAT activity in *M. galloprovincialis* and *R. philippinarum* exposed to waterborne DIC as the responses were highly tissue and species dependent. Recently, Mezzelani et al. (2018) found that antioxidant defences in *M. galloprovincialis* exposed to DIC presented only limited and not statistically significant variations between different time
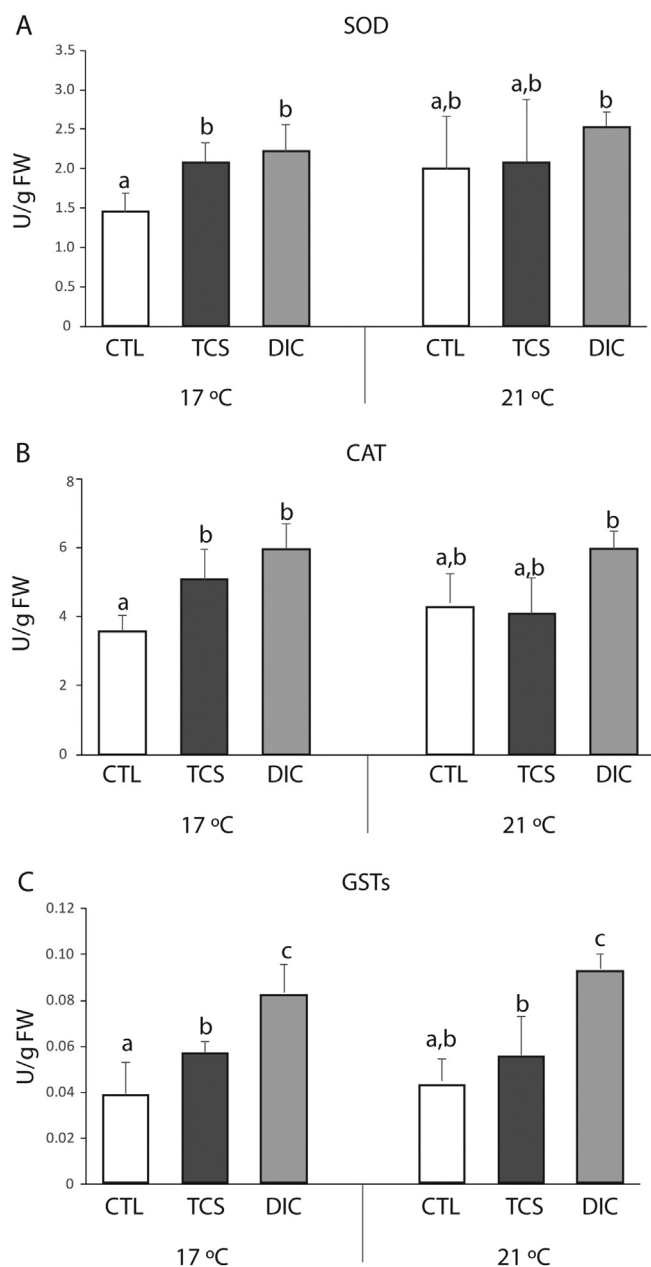
Fig. 2. A: Superoxide dismutase activity (SOD); B: catalase activity (CAT) and C: glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* under two temperatures 17 °C (natural) and 21 °C (predicted CC scenario) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 μg/L each. Values are presented as mean + standard deviation. Significant differences (p ≤ 0.05) among treatments are represented with different letters.

exposures. Overall, the present results highlight that mussels are able to activate their antioxidant defences when exposed to environmentally relevant concentrations of TCS and DIC but, and according to previously published studies, these defence responses may vary depending on the species used, tissues analysed, exposure duration, drug type and concentrations tested. Furthermore, the present study shows that antioxidant alterations were independent of temperature, indicating that increased temperature may not induce additive adverse effects on the mussel's antioxidant capacity.

Glutathione S-transferase activity (GSTs) (Fig. 2C) showed no significant differences between non-contaminated mussels at 17 °C and 21 °C. Significantly higher GSTs values were observed in contaminated mussels compared to non-contaminated ones, regardless of the temperature. Similar responses in this detoxification parameter were seen in

the groups with both of the stressors (higher temperature and PPCPs), which suggests that no additive effects occurred either. Furthermore, GSTs enzymes showed a comparable response to temperature and PPCPs exposure to antioxidant defences. The enzymes GSTs catalyse the conjugation of glutathione (GSH) to electrophilic xenobiotics, which plays an important role in the metabolism and detoxification of numerous endogenous and xenobiotic compounds, including oxidised lipids, drugs, and pollutants. They also have an antioxidant role. The present study clearly demonstrates that GSTs were involved in TCS and, more significantly, in DIC detoxification. Canesi et al. (2007) and Binelli et al. (2011) also demonstrated that TCS stimulated the activity of GSTs in the digestive gland of the marine mussel *M. galloprovincialis* and in the whole tissue of the freshwater mussel *D. polymorpha*. Regarding DIC, Schmidt et al. (2011) showed an increase of GSTs activity in *Mytilus* spp. injected with 1 and 1000 μg/L of DIC after 96 h of exposure at 10 °C, but failed to display a response in 1 μg/L of water exposure for up to 14 days at 13 °C (Schmidt et al., 2014). In line with our results, increased GSTs were also observed by Mezzelani et al. (2018) in *M. galloprovincialis* after environmentally realistic water exposures with DIC. Therefore, our study and most of the previously published data suggests that GSTs are involved in TCS and DIC detoxification in bivalves.

3.2.3. Indicators of cellular damage

Lipid peroxidation levels (LPO) (Fig. 3A) were significantly higher in non-contaminated mussels at 17 °C compared to those at 21 °C, as well as those exposed to PPCPs, except for the DIC at 21 °C group where the LPO levels were significantly higher. Protein carbonylation values (PC) (Fig. 3B) were significantly higher in control mussels at 17 °C compared to the controls at 21 °C and those exposed to TCS and DIC at the higher temperature (21 °C). The ratio between reduced and oxidised glutathione (GSH/GSSG) was significantly lower in the drug-exposed mussels under both of the temperature regimes. Moreover, this ratio in control mussels at 21 °C was lower than the controls at 17 °C. It has already been demonstrated that the toxic mechanisms of TCS and DIC involve the generation of ROS, resulting in oxidative stress (Gomez-Lechon et al., 2003; Quinn et al., 2011). Current results further demonstrate that mussels exposed to TCS at both temperatures and DIC exposed at 17 °C are able to prevent oxidative damage. This is likely to be a result of metabolic capacity decrease (ETS activity), which reduces the production of ROS via the mitochondrial respiration chain, and increased antioxidant and biotransformation enzyme defences. However, mussels exposed to warmer conditions and DIC displayed a significant increase in LPO levels, but not oxidised proteins, indicating that, although antioxidant defences and GSTs were activated, when they were exposed to warmer conditions they were not able to neutralize the excess of ROS and so lipid damage occurred. Very consistent results were obtained for GSH/GSSG levels. A significantly lower GSH/GSSG ratio was observed in contaminated mussels under both temperature regimes (17 and 21 °C) but also in the unexposed groups kept at a higher temperature. A decrease in total GSH was also reported after long term exposure to DIC in *M. galloprovincialis* (Mezzelani et al., 2018). Former studies demonstrated that mussels were able to prevent the occurrence of LPO using the protective action of SOD in *R. philippinarum* clams exposed to environmentally realistic levels of TCS (Matozzo et al., 2012). In contrast, Riva et al. (2012) showed that the freshwater mussel *D. polymorpha* exposed to the same contaminant presented a significant increase in LPO levels. Regarding DIC, Schmidt et al. (2011) demonstrated that injected DIC significantly induced LPO in mussels (*Mytilus* spp.), but no action on this biomarker was seen for water exposures of 1 μg/L exposed for up to 21 days (Schmidt et al., 2014). Quinn et al. (2011) reported enhanced LPO levels in the freshwater mussels *D. polymorpha* exposed to the same pharmaceutical. The particular observed increase of LPO levels in DIC contaminated mussels under global warming conditions may indicate that organisms that are exposed to this drug at higher temperatures are subjected to an additional stress that they are not able to combat, which primarily affects LPO levels. In the other situations, antioxidant

A LPO

B PC
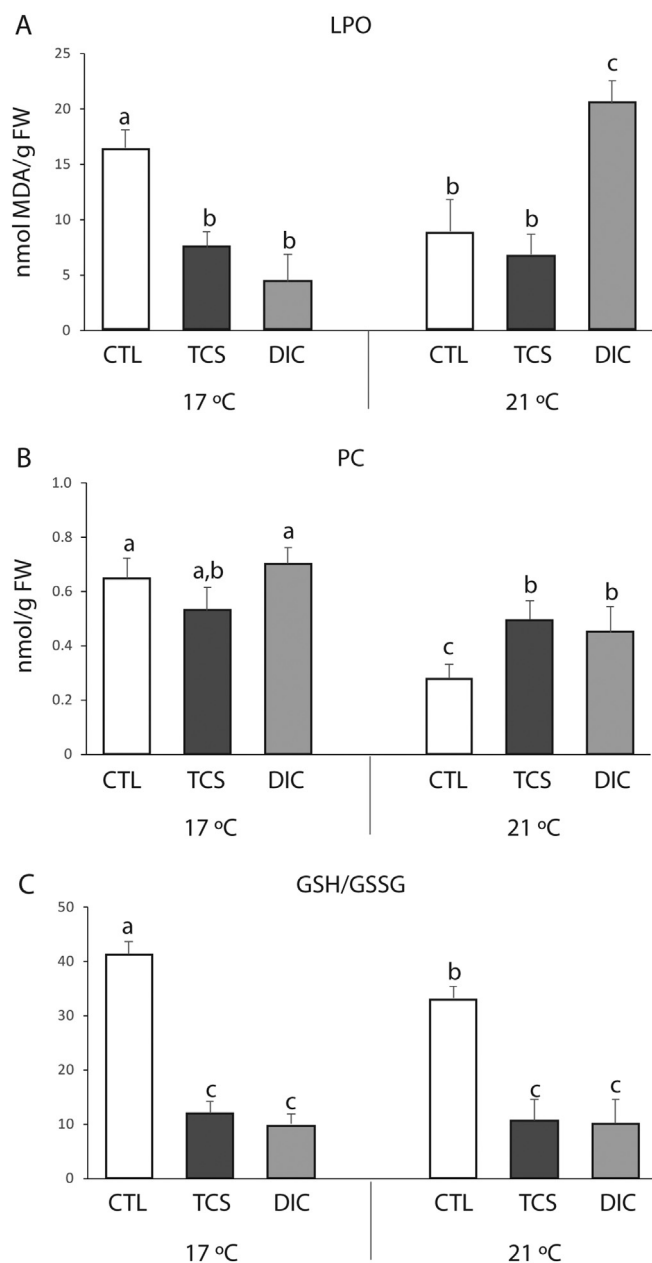
C GSH/GSSG

Fig. 3. A: Lipid peroxidation levels (LPO); B: protein carboxylation levels (PC) and C: reduced/oxidised glutathione GSH/GSSG ratio, in *Mytilus galloprovincialis* under two temperatures 17 °C (natural) and 21 °C (predicted CC scenario) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 μg/L each. Values are presented as mean + standard deviation. Significant differences (p ≤ 0.05) among treatments are represented with different letters.

defences combined with a decreased metabolism were able to prevent the occurrence of LPO in *M. galloprovincialis*. Nevertheless, consistently low GSH/GSSG values that were observed in the contaminated mussels clearly indicated that these organisms were facing oxidative stress that could compromise their biochemical and physiological performance, especially their reproduction and growth success. Our results also indicate that warmer conditions do not enhance this oxidative stress status, again highlighting that the effects of PPCPs overlap with the impact of increased temperature.

4. Conclusion

Adult mussels, *Mytilus galloprovincialis* exposed to environmentally realistic concentrations of TCS and DIC under a climate change scenario

of a 4 °C increase in temperature were able to respond to the insults by reducing their metabolic activity and increasing their antioxidant defences. Nonetheless, these responses did not prevent the occurrence of lipid peroxidation in those that were exposed to DIC at the highest temperature. Moreover, the stress situation that is caused by either enhanced temperature or PPCPs exposure caused a reduction of the GSH/GSSG ratio that may compromise the long-term response of mussels to other chemical insults. No clear synergistic effects of the two stressors (drugs and temperature) were revealed as both PPCPs caused a similar impact regardless of the exposure temperature.

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